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## SCIENTIFIC OPINION

### Scientific Opinion on Flavouring Group Evaluation 213, Revision 1 (FGE.213Rev1): Consideration of genotoxic potential for $\alpha,\beta$ -Unsaturated Alicyclic ketones and precursors from chemical subgroup 2.7 of FGE.19<sup>1</sup>

EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF)<sup>2,3</sup>

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#### ABSTRACT

The Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids of the European Food Safety Authority was requested to evaluate the genotoxic potential of 26 flavouring substances from subgroup 2.7 of FGE.19 in the Flavouring Group Evaluation 213. In the first version of FGE.213 the Panel concluded based on available genotoxicity data that a concern regarding genotoxicity could be ruled out for [FL-no: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168], but for the remaining 15 substances in subgroup 2.7 further genotoxicity data were required. Based on new submitted genotoxicity data, the Panel concluded in FGE.213Rev1 that the concern regarding genotoxicity could be ruled out for 13 substances in subgroup 2.7 [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] but not for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525].

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#### KEY WORDS

FGE.213,  $\alpha,\beta$ -Unsaturated alicyclic ketones, flavouring substances, safety evaluation, Subgroup 2.7, FGE.19

<sup>1</sup> On request from the Commission, Question No EFSA-Q-2013-00102 to 00113 and EFSA-Q-2013-00560, EFSA-Q-2013-00561, EFSA-Q-2013-00554, adopted on 10 April 2014.

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## SUMMARY

The Scientific Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (the Panel) is asked to advise the Commission on the implications for human health of chemically defined flavouring substances used in or on foodstuffs in the Member States. In particular, the Scientific Panel is asked to evaluate flavouring substances using the procedure as referred to in the Commission Regulation EC No 1565/2000.

The present revision of FGE.213, FGE.213Rev1 is due to additional genotoxicity data submitted by the Industry in response to genotoxicity data requests presented in FGE.213. New genotoxicity studies have been submitted for the five substances beta-ionone [FL-no: 07.008], maltol [FL-no: 07.014], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109].

The Flavouring Group Evaluation 213 concerns 26 substances, corresponding to subgroup 2.7 of FGE.19. Twenty-three of the substances are  $\alpha,\beta$ -unsaturated alicyclic ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] and three are precursors for such ketones [FL-no: 02.106, 09.305 and 09.525].

In the first version of FGE.213 the Panel concluded that the genotoxicity concern for ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-no: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] could be ruled out and the 11 substances could accordingly be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application was required in addition to an *in vivo* Comet assay in order to clarify the genotoxic potential. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

The remaining 13 substances (including two precursors of a ketone) [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] could not be evaluated through the Procedure. Accordingly, additional data on genotoxicity were required for representatives of these 13 substances.

The flavouring Industry has informed that it does not longer support the representative flavouring substance, piperitenone oxide [FL-no: 16.044], for which the Panel requested additional data. Since the previous version of the FGE, one additional substance has been included in subgroup 2.7, tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one [FL-no: 07.224], which is structurally related to the other substances for which the genotoxic potential could not be ruled out.

In FGE.213Rev1 the Panel has evaluated the new data submitted by the Industry in response to the data request presented in FGE.213. Based on these new data the Panel concluded that the genotoxicity concern could be ruled out for the representative substances beta-ionone [FL-no: 07.008], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089], 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] and the nine substances that they represent, being [FL-no: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305].

The Panel considered also the new data submitted for maltol [FL-no: 07.014] and concluded that for maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525] the concern for genotoxicity could not be ruled out.

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## BACKGROUND AS PROVIDED BY THE EUROPEAN COMMISSION

The use of flavouring is regulated under Regulation (EC) No 1334/2008<sup>4</sup> of the European Parliament and Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods. On the basis of article 9(a) of this Regulation an evaluation and approval are required for flavouring substances.

The Union List of flavourings and source materials was established by Commission Implementing Regulation (EC) No 872/2012<sup>5</sup>. The list contains flavouring substances for which the scientific evaluation should be completed in accordance with Commission Regulation (EC) No 1565/2000<sup>6</sup>.

EFSA concluded that a genotoxic potential of 15 of the  $\alpha,\beta$ -unsaturated alicyclic ketones and precursors in FGE.213 could not be ruled out.

Information on four representative materials have now been submitted by the European Flavour Association. These are beta-ionone [FL-no: 07.008], maltol [FL-no: 07.014], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109].

This information is intended to cover also the re-evaluation of the following eight substances from FGE.19 subgroup 2.7:

- 4-(2,6,6-trimethylcyclohexenyl)but-3-en-2-ol [FL-no: 02.106]
- Methyl-beta-ionone [FL-no: 07.010]
- beta-Isomethylionone [FL-no: 07.041]
- p-Mentha-1,4(8)-dien-3-one [FL-no: 07.127]
- 4,4a,5,6-Tetrahydro-7-methylnaphthalen-2(3H)-one [FL-no: 07.136]
- 4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one [FL-no: 07.200]
- beta-Ionyl acetate [FL-no: 09.305]
- Maltol isobutyrate [FL-no: 09.525]

Furthermore, information on one representative material, beta-damascone [FL-no: 07.083] has now been submitted by the European Flavour Association. This information is intended to cover also the re-evaluation of the following two substances.

- beta-Damascenone [FL-no: 07.108]
- trans-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)-but-2-en-1-one [FL-no: 07.224]

<sup>4</sup> Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 December 2008 on flavourings and certain food ingredients with flavouring properties for use in and on foods and amending Council Regulation (EEC) No 1601/91, Regulations (EC) No 2232/96 and (EC) No 110/2008 and Directive 2000/13/EC. OJ L 354, 31.12.2008, p. 34-50

<sup>5</sup> EC (European Commission), 2012. Commission implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10.2012, p. 1-161

<sup>6</sup> Commission Regulation (EC) No 1565/2000 of 18 July 2000 laying down the measures necessary for the adoption of an evaluation programme in application of Regulation (EC) No 2232/96. OJ L 180, 19.7.2000, p. 8-16

The Commission asks EFSA to evaluate this new information and depending on the outcome proceed to the full evaluation of the flavouring substances.

### TERMS OF REFERENCE AS PROVIDED BY THE EUROPEAN COMMISSION

The European Commission requests the European Food Safety Authority to carry out a safety assessment on the following 15 flavouring substances: 4-(2,6,6-trimethyl-1-cyclohexenyl)but-3-en-2-ol [FL-no: 02.106], beta-ionone [FL-no: 07.008], methyl-beta-ionone [FL-no: 07.010], maltol [FL-no: 07.014], beta-isomethylionone [FL-no: 07.041], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089], beta-damascenone [FL-no: 07.108], 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109], p-mentha-1,4(8)-dien-3-one [FL-no: 07.127], 4,4a,5,6-tetrahydro-7-methylnaphthalen-2(3H)-one [FL-no: 07.136], 4-(2,5,6,6-tetramethyl-1-cyclohexenyl)but-3-en-2-one [FL-no: 07.200], trans-1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-but-2-en-1-one [FL-no: 07.224], beta-ionyl acetate [FL-no: 09.305] and maltyl isobutyrate [FL-no: 09.525] in accordance with Commission Regulation (EC) N° 1565/2000.

### HISTORY OF THE EVALUATION OF FGE.19 SUBSTANCES

Flavouring Group Evaluation 19 (FGE.19) contains 360 flavouring substances from the EU Register being  $\alpha,\beta$ -unsaturated aldehydes or ketones and precursors which could give rise to such carbonyl substances via hydrolysis and/or oxidation (EFSA, 2008a).

The  $\alpha,\beta$ -unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a). The Panel noted that there were limited genotoxicity data on these flavouring substances but that positive genotoxicity studies were identified for some substances in the group.

The  $\alpha,\beta$ -unsaturated carbonyls were subdivided into subgroups on the basis of structural similarity (EFSA, 2008a). In an attempt to decide which of the substances could go through the Procedure, a (quantitative) structure-activity relationship (Q)SAR prediction of the genotoxicity of these substances was undertaken considering a number of models (DEREKfW, TOPKAT, DTU-NFI-MultiCASE Models and ISS-Local Models, (Gry et al., 2007)).

The Panel noted that for most of these models internal and external validation has been performed, but considered that the outcome of these validations was not always extensive enough to appreciate the validity of the predictions of these models for these  $\alpha,\beta$ -unsaturated carbonyls. Therefore, the Panel considered it inappropriate to totally rely on (Q)SAR predictions at this point in time and decided not to take substances through the procedure based on negative (Q)SAR predictions only.

The Panel took note of the (Q)SAR predictions by using two ISS Local Models (Benigni and Netzeva, 2007a; Benigni and Netzeva, 2007b) and four DTU-NFI MultiCASE Models (Gry et al., 2007; Nikolov et al., 2007) and the fact that there are available data on genotoxicity, *in vitro* and *in vivo*, as well as data on carcinogenicity for several substances. Based on these data the Panel decided that 15 subgroups (1.1.1, 1.2.1, 1.2.2, 1.2.3, 2.1, 2.2, 2.3, 2.5, 3.2, 4.3, 4.5, 4.6, 5.1, 5.2 and 5.3) (EFSA, 2008b) could not be evaluated through the Procedure due to concern with respect to genotoxicity. Corresponding to these subgroups, 15 Flavouring Group Evaluations (FGEs) were established: FGE.200, 204, 205, 206, 207, 208, 209, 211, 215, 219, 221, 222, 223, 224 and 225.

For 11 subgroups the Panel decided, based on the available genotoxicity data and (Q)SAR predictions, that a further scrutiny of the data should take place before requesting additional data from the Flavouring Industry on genotoxicity. These subgroups were evaluated in FGE.201, 202, 203, 210, 212, 213, 214, 216, 217, 218 and 220. For the substances in FGE.202, 214 and 218 it was concluded that a genotoxic potential could be ruled out and accordingly these substances will be evaluated using the Procedure. For all or some of the substances in the remaining FGEs, FGE.201, 203, 210, 212, 213, 216, 217 and 220 the genotoxic potential could not be ruled out.

To ease the data retrieval of the large number of structurally related  $\alpha,\beta$ -unsaturated substances in the different subgroups for which additional data are requested, EFSA worked out a list of representative

substances for each subgroup (EFSA, 2008c). Likewise an EFSA genotoxicity expert group has worked out a test strategy to be followed in the data retrieval for these substances (EFSA, 2008b).

The Flavouring Industry has been requested to submit additional genotoxicity data according to the list of representative substances and test strategy for each subgroup.

The Flavouring industry has now submitted additional data and the present FGE concerns the evaluation of these data requested on genotoxicity.

## ASSESSMENT

### 1. HISTORY OF THE EVALUATION OF THE SUBSTANCES BELONGING TO FGE.213

In the EFSA Opinion “List of  $\alpha,\beta$ -unsaturated aldehydes and ketones representative of FGE.19 substances for genotoxicity testing” (EFSA, 2008c), representative flavouring substances have been selected for FGE.19 subgroup 2.7, corresponding to FGE.213.

In the first scientific opinion on FGE.213 (EFSA, 2009), the Panel concluded that based on the data available the concern with respect to genotoxicity could be ruled out for 11 substances, [FL-no: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 7.117, 07.118, 07.119, 07.120 and 07.168]. Nine of these substances have been evaluated by the JECFA before 2000 to be of no safety concern and in accordance with Commission Regulation (EC) No 1565/2000 no further considerations is requested. The remaining two substances [FL-no: 07.047 and 07.168] will be evaluated in FGE.83Rev1 and FGE.11Rev2, respectively, using the Procedure.

For maltol [FL-no: 07.014], the Panel has requested a combined *in vivo* micronucleus and Comet assay in order to clarify the genotoxic potential. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

For the remaining 13 substances [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 07.224 and 09.305] additional data on genotoxicity were required for the representative substances, according to the Opinion of the CEF Panel on the “Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19” (EFSA, 2008b).

FGE	Adopted by EFSA	Link	No. of Substances
FGE.213	27 November 2008	<a href="http://www.efsa.europa.eu/en/efsajournal/pub/879.htm">http://www.efsa.europa.eu/en/efsajournal/pub/879.htm</a>	26
FGE.213Rev1	10 April 2014		26

The present FGE.213 Revision 1 (FGE.213Rev1) includes the assessment of additional genotoxicity data submitted by Industry (IOFI, 2012; IOFI, 2013) in reply to a data request presented in FGE.213 (EFSA, 2009).

The new data submitted concerns five of the original six representative substances requested by the Panel (EFSA, 2008c), namely beta-ionone [FL-no: 07.008], maltol [FL-no: 07.014], beta-damascone [07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109], (Table 1).

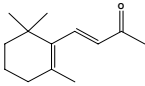

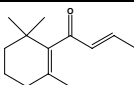
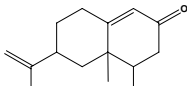
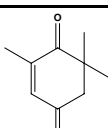
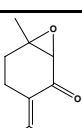
The flavouring Industry has informed that it does not longer support the representative flavouring substance, piperitenone oxide [FL-no: 16.044], for which the Panel requested additional data. However, since piperitenone oxide was a self-representative substance, this will not affect the evaluation of the remaining substances in FGE.213Rev1.

Since the previous version of the FGE, one additional substance has been included in subgroup 2.7, tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one [FL-no: 07.224], which is structurally related to the other substances for which the genotoxic potential could not be ruled out.

The new data submitted for the five representative substances are described and evaluated in Section 4 of the present revision. Sections 2 and 3 report the same information that was present in the earlier version of FGE.213.



**Table 1:** Representative substances for subgroup 2.7 of FGE.19

FL-no JECFA-no	EU Register name	Structural formula	Comments
07.008 389	beta-Ionone		<i>In vitro</i> assays in bacteria and mammalian cells submitted
07.014 1480	Maltol		<i>In vitro</i> assays in bacteria and mammalian cells and an <i>in vivo</i> combined Comet and micronucleus assay submitted
07.083 384	beta-Damascone		<i>In vitro</i> assays in bacteria and mammalian cells and an <i>in vivo</i> combined Comet and micronucleus assay submitted
07.089 1398	Nootkatone		<i>In vitro</i> assays in bacteria and mammalian cells submitted
07.109 1857	2,6,6-Trimethylcyclohex-2-en-1,4-dione		<i>In vitro</i> assays in bacteria and mammalian cells submitted
16.044 1574	Piperitenone oxide		No longer supported by Industry and no data submitted

## 2. Presentation of the Substances in Flavouring Group Evaluation 213

### 2.1. Description

The Flavouring Group Evaluation 213 (FGE.213) concerned 26 substances (Table 2), corresponding to subgroup 2.7 of FGE.19. Twenty-three of the substances are  $\alpha,\beta$ -unsaturated alicyclic ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] ([FL-no 16.044] is no longer supported by Industry and one new substance ([FL-no 07.224] has been included in Revision 1) and three are precursors for such ketones [FL-no: 02.106, 09.305 and 09.525]. Two of these substances [FL-no: 02.106 and 09.305] are precursors of the ketone beta-ionone [FL-no: 07.008] and one [FL-no: 09.525] is a precursor of the ketone maltol [FL-no: 07.014]. Ten of the ketones have the possibility for keton-enol tautomerism [FL-no: 07.056, 07.057, 07.075, 07.076, 07.080, 07.117, 07.118, 07.119, 07.120 and 07.168]. Based on experimental evidence for other diketones it is anticipated that the enol is the predominant form.

Twenty-two of the substances in the present FGE.Rev1 (including the new substance ([FL-no 07.224], excluding ([FL-no 16.044]) have formerly been evaluated by the JECFA (JECFA, 1999; JECFA, 2001; JECFA, 2006a; JECFA, 2006b; JECFA, 2009a), a summary of their current evaluation status by the JECFA is given in Table 3.

As the  $\alpha,\beta$ -unsaturated aldehyde and ketone structures are structural alerts for genotoxicity (EFSA, 2008a) the available data on genotoxic or carcinogenic activity for the 26 unsaturated alicyclic ketones and precursor in subgroup 2.7 will be considered in this FGE.

The Panel has also taken into consideration the outcome of the predictions from five selected (Q)SAR models (Benigni & Netzeva, 2007a; Gry et al., 2007; Nikolov et al., 2007) on 22 ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168 and 07.200]. The 22 ketones and their (Q)SAR predictions are shown in Table 4.

### 3. Toxicity<sup>7</sup>

#### 3.1. (Q)SAR Predictions

In Table 4 the outcomes of the (Q)SAR predictions for possible genotoxic activity in five *in vitro* (Q)SAR models (ISS-Local Model-Ames test, DTU-NFI-MULTICASE-Ames test, Chromosomal aberration test (CHO), Chromosomal aberration test (CHL), and Mouse lymphoma test) are presented.

Maltol [FL-no: 07.014], ethyl maltol [FL-no: 07.047] and nootkatone [FL-no: 07.089] were predicted positive with the MultiCASE model on chromosomal aberrations in CHL cells. All other predictions were negative or the substances were out of domain.

#### 3.2. Genotoxicity Studies

In subgroup 2.7 there are studies available for four substances. For maltol [FL-no: 07.014] eight *in vitro* and three *in vivo* studies have been evaluated. For ethyl maltol [FL-no: 07.047] two *in vitro* and one *in vivo* study were evaluated. Numbers of evaluated *in vitro* studies concerning beta-ionone [FL-no: 07.008] and 3-methylcyclopentan-1,2-dione [FL-no: 07.056] were two and one, respectively.

Study validation and results are presented in Tables 5 and 6.

In studies which were considered valid, the following results were obtained:

Maltol induced gene mutations in bacteria (Bjeldanes and Chew, 1979) and sister chromatid exchanges in human lymphocytes (Jansson et al., 1986). *In vivo*, maltol induced micronuclei in mouse bone marrow after intraperitoneal application (Hayashi et al., 1988). Negative results were obtained in a sex-linked recessive lethal mutation assay in *Drosophila* (Mason et al., 1992). However, the micronucleus assay is considered more relevant than the *Drosophila* assay.

Ethyl maltol induced gene mutations in bacteria (Bjeldanes and Chew, 1979).

A negative result was obtained with beta-ionone in a gene mutation assay in bacteria (Mortelmans et al., 1986).

The validity of other studies was limited or could not be evaluated.

#### 3.3. Carcinogenicity Studies

In a combined study of developmental toxicity and carcinogenicity, three successive generations of male and female Charles River CD-COBS rats received 3-ethyl-2-hydroxy-2-cyclopenten-1-one (due to keto-enol tautomerism this substance can exist as two isomers; the keto-isomer is 3-ethylcyclopentan-1,2-dione [FL-no: 07.057], a synonym for the keto-isomer is ethylcyclopentenolone) in the basal diet at doses of 0 (untreated control), 0 (propylene glycol control), 30, 80 or 200 mg/kg body weight (bw) per day. The F1 generation was initially exposed in utero, subsequently via the

<sup>7</sup> The data presented in Section 3 is cited from the first version of the present FGE.213. These data are the basis for the conclusions in FGE.213 requesting additional genotoxicity data.

dams' milk until weaning, and then treated for two years and bred twice (at days 99 and 155). In the F1 generation, there were 100 animals of each sex in the untreated control group and 50 of each sex in the propylene glycol control and 3-ethyl-2-hydroxy-2-cyclopenten-1-one-treated groups. Survival, clinical symptoms, food consumption, reproductive performance, and haematological and clinical chemical parameters were not adversely affected. Gross pathological and histopathological examination revealed no significant treatment-related effects. The incidence of benign or malignant tumours in treated animals was similar to that in controls. The No Observed Effect Level (NOEL) was 200 mg/kg bw per day (King et al., 1979).

The Panel concluded that 3-ethyl-2-hydroxy-2-cyclopenten-1-one (3-ethylcyclopentan-1,2-dione [FL-no: 07.057]) was not carcinogenic in rats under the study conditions.

Groups of 25 male and female rats were fed for two years on diets containing ethyl maltol [FL-no: 07.047] calculated to deliver 0, 50, 100 and 200 mg ethyl maltol/kg bw/day. No abnormalities were seen as regards survival, clinical appearance, growth rate or food consumption, clinical chemistry, haematology and urinalysis. No histopathological changes and no increases in neoplasms were seen after the treatment with ethyl maltol (Gralla et al., 1969).

Study validation and results are presented in Table 7.

The Panel noted that this study was performed before OECD test guidelines 451/453 (1981) have been established and it does not meet the criteria of these OECD test guidelines with respect to the number of animals. However, the Panel concluded that ethyl maltol was not carcinogenic in rats in this study.

### 3.4. Conclusion on Genotoxicity and Carcinogenicity

For the substances of this group, the applicability of the (Q)SAR models is very limited since many substances were out of domain in the ISS model and the MultiCASE models.

Two substances [FL-no: 02.106 and 09.305] are precursors of beta-ionone [FL-no: 07.008] and therefore, the conclusions for these two precursors could be based on the conclusions drawn for the corresponding ketone [FL-no: 07.008]. Maltol isobutyrate [FL-no: 09.525] is a precursor of maltol [FL-no: 07.014], and accordingly, the conclusion for maltol isobutyrate could be based on the conclusion drawn for maltol.

Maltol and ethyl maltol were considered separately because in contrast to the other substances in this subgroup they contain a ring-oxygen atom.

There is a carcinogenicity study on ethyl maltol [FL-no: 07.047] in rats. Although the number of animals per group were lower than suggested in OECD guidelines they were in accordance with the standards at the time the study was performed and the Panel concluded that the result could overrule the mutagenicity observed with ethyl maltol in bacteria but not the mutagenicity observed with maltol [FL-no: 07.014] *in vitro* and *in vivo*. Since the micronuclei induced by maltol in mice were analysed after intraperitoneal application, a micronucleus assay after oral application is required in addition to an *in vivo* Comet assay in order to clarify the genotoxic potential of maltol. A combination of the micronucleus assay and the Comet assay in a single study would also be acceptable. The result of these assays would also be applicable to maltol isobutyrate [FL-no: 09.525] which is a precursor of maltol.

No carcinogenicity was observed with 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] in rats. This substance was considered representative for nine substances [FL-no: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080]. Therefore, the Panel concluded that the structural alert for genotoxicity is overruled for 3-ethyl-2-hydroxy-2-cyclopenten-1-one [FL-no: 07.057] as well as for the nine structurally related substances.

For the 13 remaining substances (including two precursors of a ketone) [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] a genotoxic potential could not be ruled out since only one valid negative bacterial genotoxicity study on [FL-no: 07.008] is available for these substances.

### 3.5. Conclusions

The Panel concluded that ethyl maltol [FL-no: 07.047], 3-ethylcyclopentan-1,2-dione [FL-no: 07.057] and the nine structurally related substances [FL-no: 07.117, 07.118, 07.119, 07.120, 07.056, 07.168, 07.075, 07.076 and 07.080] can be evaluated through the Procedure.

For maltol [FL-no: 07.014], a micronucleus assay after oral application is required in addition to an *in vivo* Comet assay in order to clarify the genotoxic potential. A combination of the micronucleus assay and the Comet assay in a single study would also be acceptable. The outcome would also be applicable to maltyl isobutyrate [FL-no: 09.525].

The remaining 13 substances (including two precursors of a ketone) [FL-no: 02.106, 07.008, 07.010, 07.041, 07.083, 07.089, 07.108, 07.109, 07.127, 07.136, 07.200, 09.305 and 16.044] cannot presently be evaluated through the Procedure. Additional data on genotoxicity are requested for representative substances of this subgroup according to the opinion of the Panel on the Genotoxicity Test Strategy for Substances Belonging to Subgroups of FGE.19 (EFSA, 2008b).

## 4. Industry Response to Data Requested in FGE.213

### 4.1. Presentation of the Additional Data

Based on Panel requirements published in FGE.213 (EFSA, 2009), additional data have been provided by the Industry for the representative substances: beta-ionone [FL-no: 07.008], maltol [FL-no: 07.014], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089], and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]

The present FGE.213, Revision 1 (FGE.213Rev1), includes the assessment of these additional genotoxicity data. The study types provided are shown below:

Substance / study type	Bacterial Mutation	<i>In Vitro</i> Micronucleus	<i>In Vivo</i> Micronucleus combined with Comet
beta-Ionone [FL-no: 07.008]	(Ballantyne, 2011)	(Stone, 2011a)	
Maltol [FL-no: 07.014]	(Ballantyne, 2012)	(Whitwell, 2012)	(Beevers, 2013a)
beta-Damascone [FL-no: 07.083]	(Bowen, 2011b)	(Stone, 2012)	(Beevers, 2013b; Beevers, 2013c)
Nootkatone [FL-no: 07.089]	(Marzin, 1998)	(Stone, 2011b)	
2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]	(Bowen, 2011a)	(Lloyd, 2011)	

## 4.2. *In vitro* data

### 4.2.1. *Bacterial Reverse Mutation Assay*

#### *beta-Ionone [FL-no: 07.008]*

beta-Ionone [FL-no: 07.008] was tested in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Ballantyne, 2011). In the first experiment, the concentrations were 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate of beta-ionone and the plate incorporation methodology was used. Toxicity ranging from slight thinning of the background lawn to complete killing of the tester strains was observed at 1000 and/or 5000 µg/plate for all tester strains in the absence and presence of S9-mix. In the second experiment, the concentrations were 10.24, 25.6, 64, 160, 400 and 1000 µg/plate and the treatments in the presence of S9-mix used the pre-incubation method. Toxicity ranging from thinning of the background lawn and/or reduction in revertant numbers to complete killing of the tester bacteria occurred in all strains at 1000 µg/plate in the absence and presence of S9-mix and was also seen down to 160 and/or 400 µg/plate for some individual strains. The study design complied with current recommendations and an acceptable top concentration was achieved. There was clearly no evidence of any mutagenic effect induced by beta-ionone in any of the strains, either in the absence or presence of S9-mix.

#### *Maltol [FL-no: 07.014]*

Maltol [FL-no: 07.014] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Ballantyne, 2012). In the first experiment, the concentrations were 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate of maltol and the plate incorporation methodology was used. Toxicity in the form of reduction of the number of revertants in the tester strain TA102 was observed at 200 µg/plate and above in the presence of S9-mix and 1000 and 5000 µg/plate in the absence of S9-mix. In the second experiment, the concentrations were 51.2, 128, 320, 800, 2000 and 5000 µg/plate and the treatments in the presence of S9-mix used the pre-incubation method in all strains. In tester strain TA102 an additional lower concentration of 20.48 µg/plate was incorporated into the testing protocol in both the absence and presence of S9 to more carefully assess the toxicity observed in Experiment 1. Toxicity in the form of thinning of the background lawn and/or reduction in numbers of revertants occurred at the 5000 µg/plate concentration in strain TA102 in the absence and presence of S9-mix and in strain TA100 only in the presence of S9-mix. The study design complied with current recommendations and an acceptable top concentration was achieved. There was no evidence of any mutagenic effect induced by maltol in any of the strains, either in the absence or presence of S9-mix.

#### *beta-Damascone [FL-no: 07.083]*

An Ames assay was conducted in *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537, and TA102 to assess the mutagenicity of beta-damascone (purity: 95 %), both in the absence and in the presence of metabolic activation by S9-mix, in three separate experiments (Bowen, 2011b). The assay was performed according to OECD Guideline 471 (1997a) and according to GLP principles.

An initial experiment was carried out both in the absence and presence of S9-mix activation in all five strains, using final concentrations of beta-damascone between 0.32 - 5000 µg/plate (0.32, 1.6, 8, 40, 200, 1000, 5000 µg/plate), plus negative (solvent) and positive controls. Evidence of toxicity was observed through thinning of the background lawn to complete killing at 1000 µg/plate and above for strains TA1535, TA1537 and TA102 and/or 5000 µg/plate for strains TA98 and TA100 in the absence and presence of S9-mix. In the second experiment the highest concentration was retained for strains TA98 and TA100 in the absence and presence of S9-mix. In all other tester strains, the highest dose was reduced to 2500 µg/plate based on toxicity observations. In addition, more narrow concentration intervals were used, starting at either 78.13 µg/plate or 156.3 µg/plate (78.13, 156.3, 312.5, 625, 1250, 2500 µg/plate). The standard plate incorporation assay was used in the first experiment but a pre-

incubation step with S9-mix activation treatment was added in the second experiment to increase the chance of detecting a positive response. Evidence of toxicity was observed in TA98 at 625 µg/plate in the presence of S9-mix in addition to strains TA1535, 1537 and TA 102 in the absence and presence of S9-mix, at 1250 µg/plate and above in strain TA98 in the absence of S9-mix and TA100 in the presence of S9-mix and TA100 in the absence of S9-mix at 2500 µg/plate and above.

The third experiment was conducted using strain TA98 in the presence of S9-mix activation using the pre-incubation method. The maximum test concentration was reduced to 1250 µg/plate based on toxicity observed in the previous experiments. In addition, more narrow concentration intervals were used, covering 19.53 to 1250 µg beta-damascone/plate (19.53, 39.06, 78.13, 156.3, 312.5, 625 and 1250 µg/plate). Evidence of toxicity was observed at the highest four concentrations in strain TA98 in the presence of S9-mix. In all three experiments, no statistically significant increases in revertant numbers were observed at any concentration, in any of the strains, either in the presence or absence of S9-mix activation.

The Panel agreed with the conclusion of the study authors that beta-damascone did not induce mutations in five strains of *Salmonella typhimurium*, when tested under the conditions of this study.

#### *Nootkatone [FL-no: 07.089]*

Nootkatone [FL-no: 07.089] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence or presence of S9-mix (Marzin, 1998). A preliminary toxicity test to identify appropriate concentrations for the mutagenicity assays was performed in the absence and presence of S9-mix, and cytotoxicity was observed at 50 µg/plate in the absence of S9-mix and at 150 µg/plate in the presence of S9-mix. In the first mutagenicity experiment using plate incorporation methodology the concentrations tested were 0.5, 1.5, 5, 15 and 50 µg/plate in the absence of S9-mix metabolic activation and 1.5, 5, 15, 50 and 150 µg/plate in the presence of S9-mix. In the second experiment the plate incorporation method was used in absence of S9 and the concentrations were 0.5, 1.5, 5, 15 and 50 µg/plate. While the pre-incubation method was used in the presence of S9-mix and the concentrations were 0.5, 1.5, 5, 15, 50 and 150 µg/plate. Thus, the study design complied with current recommendations and an acceptable top concentration was achieved. There was no evidence of any mutagenic effect induced by nootkatone in any of the strains, either in the absence or presence of S9-mix.

#### *2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]*

2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] was tested in *S. typhimurium* strains TA98, TA100, TA1535, TA1537 and TA102 in the absence and presence of S9-mix (Bowen, 2011a). In the first experiment, the concentrations tested were 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate and plate incorporation methodology was used. In the second experiment, the concentrations were 156.3, 312.5, 625, 1250, 2500 and 5000 µg/plate of 2,6,6-trimethylcyclohex-2-en-1,4-dione and treatments in the presence of S9-mix used the pre-incubation method. The test chemical elicited evidence of cytotoxicity in the form of background lawn thinning or marked reduction of the number of revertants in experiment 1 at 1000 and/or 5000 µg/plate in strains TA102 and TA1535 in the presence of S9-mix and in experiment 2 at 2500 and/or 5000 µg/plate in strain TA102 in the absence and presence of S9-mix. Thus, the study design complied with current recommendations and an acceptable top concentration was achieved. There was no evidence of any mutagenic effect induced by 2,6,6-trimethylcyclohex-2-en-1,4-dione in any of the strains, either in the absence or presence of S9-mix.

Summary of the Bacterial Reverse Mutation Assay for all the substances are reported in Table 8.



#### 4.2.2. *Micronucleus Assays*

##### *beta-Ionone [FL-no: 07.008]*

beta-Ionone [FL-no: 07.008] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat liver S9-mix fraction as an *in vitro* metabolising system. Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with 0, 30, 50 or 60 µg/ml of beta-ionone in the absence of S9-mix and 0, 80, 100 or 120 µg/ml in the presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 52 % and 59 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 5, 15 and 17.50 µg/ml of beta-ionone in the absence of S9-mix with no recovery period. The top concentration induced 58 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate were scored for micronuclei. Thus, the study design complies with current recommendations (OECD Guideline 487), and acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study. Treatment of cells with beta-ionone for 3 hours with a 21 hours recovery period showed an increase in the frequency of MNBN cells in one single replicate at the concentration of 30 and 120 µg/ml (0.9 % and 1.5 %, respectively) in the absence and presence of S9-mix, respectively. At 30 µg/ml, the lowest concentration tested in the absence of S9-mix, the increase in the frequency of MNBN cells was slightly above the 95 % confidence interval of the historical control range (0.2 - 0.8 %). Also in the presence of S9-mix, one replicate of the lowest concentration tested (80 µg/ml) had an increase in the frequency of MNBN cells at the upper limit of the 95 % confidence interval of the historical control range (0.10 - 1.10 %) but did not reach statistical significance. To ensure that these single occurrences are random an additional 1000 binucleate cells were scored from the concurrent controls, 80 and 120 µg/ml cultures. The scoring of further cells resulted in overall mean frequencies of MNBN cells that were not significantly different from concurrent controls and fell below the upper 95 % confidence interval of the normal control range (recalculated due to change of stain), and therefore showed that the earlier increases were due to chance. It was concluded that beta-ionone [FL-no: 07.008] did not induce micronuclei up to toxic concentrations when assayed in cultured human peripheral lymphocytes for 3 + 21 hours in the absence and presence of S9-mix or when incubated for 24 + 0 hours in the absence of S9-mix (Stone, 2011a).

##### *Maltol [FL-no: 07.014]*

Maltol [FL-no: 07.014] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat liver S9-mix fraction as an *in vitro* metabolising system (Whitwell, 2012). Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with 0, 400, 800 or 1262 µg/ml of maltol, the latter being equivalent to 10 mM, in the absence and presence of S9-mix. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 24 % and 19 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 125, 200 and 300 µg/ml of maltol in the absence of S9-mix with no recovery period. The top concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment, and 1000 binucleate cells per replicate (i.e. 2000 cells per concentration) were scored for micronuclei. Thus, the study design complies with current recommendations (OECD Guideline 487), and acceptable top concentrations, either 10 mM or 50 - 60 % toxicity, were achieved in all parts of the study. A statistically significant increase in the occurrence of MNBN cells was observed following 3 + 21 hours treatment in the presence of S9-mix at the two highest concentrations scored. Statistically significant and concentration-dependent increases in MNBN were seen in the 3 + 21 hours treatment groups in the absence of S9-mix, but it was noted that the increases at the two highest concentrations scored only exceeded historical control ranges in one of the two replicate cultures. No increases were observed in the frequency of MNBN in cells that received continuous (24 + 0 hours) treatment, but due to the cytotoxicity of maltol, lower concentrations were analysed. To further investigate these observations, fluorescence in situ hybridisation (FISH) analysis using human pan-centromeric probes

was conducted to assess whether the mechanism of action could be attributed to chromosome loss (aneuploidy) or chromosome breakage (clastogenicity). Slides were prepared from the two highest concentrations (800 and 1262 µg/ml) in the absence and presence of S9-mix. The FISH analysis revealed that following maltol treatment the majority (69 - 76 %) of micronuclei did not contain a centromere. The Panel concluded that maltol induced micronuclei *in vitro* in cultured human peripheral blood lymphocytes in the presence of rat liver metabolic activation (S9-mix) via a clastogenic mechanism of action (Whitwell, 2012). However, the Panel considered that the results observed in the absence of S9 were equivocal due to the fact that the increases observed (which were statistically significantly different from concurrent solvent control) were not reproduced in replicate cultures.

*beta-Damascone [FL-no:07.083]*

beta-Damascone (purity: 95 %) was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9 fraction as an *in vitro* metabolising system (Stone, 2012). Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with a large range of concentrations from 2 to 30 µg/ml. For the treatment of 3 hours with a 21 hour recovery period, the concentrations of beta-damascone at 8, 16 and 22 µg/ml or at 12, 16, 18 µg/ml were retained for MN numeration, in the absence or in the presence of S9-mix respectively. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 59 % and 51 %, respectively. Thus, the study design complies with OECD Guideline 487 and follows GLP principles.

In a parallel assay, cells were treated for 24 hours (with no recovery period) in the absence of S9-mix with a large range of concentrations from 1 to 15 µg/ml and the concentrations of 6, 8 and 9 µg/ml of beta-damascone were retained for MN numeration. The top concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487, 2010), and acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study.

Treatment of cells with beta-damascone for 3 + 21 hours in the presence of S9-mix showed a statistically significant concentration-dependent increase in the induction of MNBN cells with 0.55, 2.10 and 2.70 % MNBN cells vs. 0.35 % in the concurrent control and 0.1 to 1.1 % for the historical controls.

Treatment of cells with beta-damascone for 3 + 21 and 24 + 0 hours in the absence of S-9 resulted in sporadic increases in MNBN frequency. These increases were only observed in single replicates and were not concentration related. Therefore, the effect of beta-damascone was further investigated through the scoring of additional cells (2 more replicates of 1000 cells each) from the affected concentrations and concurrent controls.

Treatment of cells, in the absence of S9-mix, for 3 + 21 hours induced a statistically significant increase in the frequency of MNBN cells at 8 and 22 µg/ml (0.80 % and 0.93 %, respectively) compared to concurrent control (0.38 %), but not at the mid dose of 16 µg/ml (0.53 % MNBN cells). The frequency of MNBN cells exceeded the historical controls (0.2 - 0.8 %) in 3 out of 4 replicates at the highest concentration tested (22 µg/ml). Treatment of cells for 24 hours with no recovery period in the absence of S9-mix showed statistically significant increase in the frequency of MNBN cells at the mid dose of 8 µg/ml (0.95 % MNBN cells) when compared to concurrent control (0.40 %) with no correlation to concentration. The frequency of MNBN cells exceeded the historical controls (0 - 1.1 %) only in 1 replicate at 8 µg/ml.

The authors considered that this result reaffirmed the sporadic nature of the induction of MNBN cells in the absence of S9-mix. It was concluded that the treatment with beta-damascone for 3 + 21 hours or



24 + 0 hours (in the absence of S9-mix) induced sporadic increases in MNBN cells when compared to concurrent controls and not concentration related, therefore the results were considered equivocal. In the same test system, beta-damascone did induce micronuclei in cultured human peripheral blood lymphocytes following 3+21 hours treatment in the presence of S9-mix (Stone, 2012). The Panel noted that after the new reading of slides the increase of MNBN cells frequency was still statistically significant even at weak cytotoxic levels.

Therefore, the Panel concluded that beta-damascone is genotoxic in the *in vitro* micronucleus assay on human lymphocytes with metabolic activation and equivocal without metabolic activation.

#### *Nootkatone [FL-no: 07.089]*

Nootkatone [FL-no: 07.089] was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9-mix fraction as an *in vitro* metabolising system (Stone, 2011b). Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with 0, 50, 70 or 80 µg/ml of nootkatone in the absence of S9-mix and 0, 160, 180 and 185 µg/ml in the presence of S9-mix, respectively. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 60 and 58 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 10, 15, 22 and 24 µg/ml of nootkatone in the absence of S9-mix with no recovery period. The top concentration induced 62 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate (i.e. 2000 cells per dose) were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487) and acceptable levels of cytotoxicity were achieved at the top concentrations used in all parts of the study. No evidence of chromosomal damage or aneuploidy was observed as frequencies of MNBN cells were not significantly different from concurrent controls and fell within historical control ranges for all treatments with nootkatone in the presence or absence of S9-mix metabolic activation (Stone, 2011b).

#### *2,6,6-Trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]*

2,6,6-Trimethylcyclohex-2-en-1,4-dione was evaluated in an *in vitro* micronucleus assay in human peripheral blood lymphocytes for its ability to induce chromosomal damage or aneuploidy in the presence and absence of rat S9-mix fraction as an *in vitro* metabolising system (Lloyd, 2011). Cells were stimulated for 48 hours with phytohaemagglutinin to produce exponentially growing cells and then treated for 3 hours (followed by 21 hours recovery) with 0, 500, 1000 or 1522 µg/ml of 2,6,6-trimethylcyclohex-2-ene-1,4-dione in the absence of S9-mix and 0, 1000, 1250 and 1522 µg/ml in the presence of S9-mix, respectively, the top concentration being equivalent to 10 mM. The levels of cytotoxicity (reduction in replication index) at the top concentrations were 3 % and 9 %, respectively. In a parallel assay, cells were treated for 24 hours with 0, 300, 420 and 550 µg/ml of 2,6,6-trimethylcyclohex-2-ene-1,4-dione in the absence of S9-mix with no recovery period. The top concentration induced 57 % cytotoxicity. There were 2 replicate cultures per treatment and 1000 binucleate cells per replicate (i.e. 2000 cells per concentration) were scored for micronuclei. The study design complies with current recommendations (OECD Guideline 487), and acceptable top concentrations, either 10 mM or 50 - 60 % toxicity, were achieved in all parts of the study. No evidence of chromosomal damage or aneuploidy was observed as frequencies of MNBN cells were not significantly different from concurrent controls and fell within historical ranges for all 2,6,6-trimethylcyclohex-2-ene-1,4-dione treatments in the presence or absence of S9-mix metabolic activation (Lloyd, 2011).

The results of *in vitro* micronucleus studies are summarised in Table 8.

### 4.3. Genotoxicity *in vivo* data

#### 4.3.1. *In vivo* Combination Assay (Comet + Micronucleus)

Since no positive results were seen in either the bacterial mutation assay or *in vitro* micronucleus tests with beta-ionone [FL-no: 07.008], nootkatone [FL-no: 07.089] or 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109], no *in vivo* follow-up testing was required. To address the effects seen in the *in vitro* micronucleus assay with maltol [FL-no: 07.014] and beta-damascone [FL-no: 07.083] a combination assay comprising of a liver Comet assay and an *in vivo* micronucleus assay in rat, after oral application, was performed to further assess the genotoxic potential for both substances. The results are summarised in Table 9.

##### *Maltol [FL-no: 07.014]*

Maltol was evaluated in an *in vivo* micronucleus assay and liver Comet assay in male Han Wistar (HsdHan:WIST) rats, 6 rats per dose group (Beevers, 2013a). The rats were administered 3 doses of 70, 350 and 700 mg/kg bw of maltol by oral gavage at time 0, 24 and 45 hours. Rats were killed and sampled at 48 hours post the initial dose. The maximum tolerable dose was estimated to be 700 mg/kg bw/day based on a range finding study at doses of maltol of 360, 500, 700, 1000, 1500 and 2000 mg/kg bw/day. Clinical observations (piloerection, ataxia, bradypnoea) and mortalities were observed from dose-level of 1000 mg/kg bw/day. For the micronucleus assay 2000 polychromatic erythrocytes (PCE) per rat were scored. The negative control had a normal, low frequency (0.11 %) of micronucleated polychromatic erythrocytes (MNPCE) and a ratio of 53.7 % PCE. The positive control group resulted in a significant increase in MNPCEs (1.58 %) accompanied by some bone marrow toxicity (29.57 % PCE). Although an individual rat in the 700 mg/kg maltol dose group showed a frequency of 9 MNPCE, which resulted in significant heterogeneity in this dose group, this was considered an outlying data point due to the other 5 rats in the group exhibiting normal control level MNPCE frequencies (Beevers, 2013a). Overall, the results showed that there were no statistically significant increases in micronucleus frequency for any dose group after oral treatment with maltol when compared to the vehicle control group. However, in the main experiment, at the dose levels selected, no clinical signs and bone marrow toxicity were observed in any animal in the maltol-treated groups which may reflect the possibility that the bone marrow and liver were not exposed.

In order to clarify this issue, the Panel requested plasma analysis for proof of exposure. Plasma for assay has been obtained from two satellite groups of male animals (3 + 3 animals) dosed with maltol by oral gavage at 700 mg/kg bw/day, during conduction of the main study (Beevers, 2013a). Plasma obtained from 0.5 ml blood drawn from the jugular vein from each animal was frozen in the event that analysis for proof of exposure and toxicokinetics were required. All doses of maltol were given as three administrations, at 0, 24 and 45 hours. A number of three samples of plasma were obtained from one group of animals at 0.5, 2 and 8 hours and three samples from the other group at 1, 4 and 24 hours from the last administration. Analysis of maltol in plasma was performed using a gas chromatography with mass selective detection (GC-MSD) method. From an analytical point of view, the Panel considered the employed approach that was based on the use of ethylmaltol as internal standard as sufficient. Results showed a marked inconsistency between sampling times and animals. In samples collected at 0.5, 2 and 8 hours from last administration maltol was found in 2 out of 3 satellite animals at plasma concentrations of 265 - 283 µg/ml after 0.5 and 2 hours but not longer detectable after 8 hours. In the plasma of the third animal maltol was not detectable at any time. On the other hand, in samples from another animal group (n = 3) collected at 1, 4 and 24 hours from last administration, maltol was found at levels of 75 - 106 µg/l after 1 hour in all 3 animals and not longer detectable after 4 and 24 hours (Mallinson and Hough, 2014). The authors concluded that results obtained provide evidence that maltol is present in plasma shortly after dosing. However, the Panel did not agree with this conclusion and it considered the results of the bioanalytical study as inconclusive.

In the combined Comet assay, liver of rats were removed at 48 hours after the first dose (i.e. 3 hours after the final dose), cut into small pieces and forced through a bolting cloth. Single cell suspensions

were embedded in low melting point agarose on slides and lysed. The DNA was unwound and subjected to electrophoresis at pH > 13 and then neutralised according to standard techniques. For each animal, 100 cells (50 cells/slide from 2 slides) were scored for comets (tail intensity and tail moment) using commercial image analysis equipment.

The Comet assay did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis and the groups treated with maltol showed mean % tail intensities and tail moments that were similar to vehicle controls and fell within historical control ranges. The positive control group treated with ethyl methanesulphonate (EMS) showed significant increases in both parameters (Beevers, 2013a).

Considering that maltol has been shown to induce micronuclei in mouse bone marrow after intraperitoneal injection, the Panel concludes that negative findings observed in the combined bone marrow micronucleus test and Comet assay in the liver of treated rats could not rule out the concern for genotoxicity for maltol since the data provided to prove systemic availability were considered inconclusive due to the inconsistency of the data.

#### *beta-Damascone [FL-no:07.083]*

A combined *in vivo* micronucleus assay/liver Comet assay was performed after oral application of beta-damascone (purity: 95.6 %) to further assess the genotoxic potential of beta-damascone and damascenes more generally. The results are summarised in Table 9. beta-Damascone was evaluated in an *in vivo* micronucleus assay and liver and duodenum Comet assay in groups of 6 male Han Wistar (HsdHan:WIST) rats per dose group (Beevers, 2013c). Based on a range finding study, 500 mg/kg/day was considered an appropriate estimate of the MTD because the doses of 750 mg/kg/day and above induced moderate to severe clinical signs of toxicity, which included piloerection, decreased activity, hunched posture and abnormal breathing. The rats were administered 3 doses of 125, 250 and 500 mg/kg bw of beta-damascone by oral gavage at time 0, 24 and 45 hours. The rats were sacrificed and sampled at 48 hours post the initial dose.

Animals administered beta-damascone showed clear findings during pathological analysis. Hepatocytes vacuolation was present in animals given 500 mg/kg/day, and was characterised by scattered, occasionally shrunken hepatocytes with perinuclear cytoplasmic eosinophilia and peripheral cytoplasmic vacuolation. Single cell necrosis was present in a single animal given 500 mg/kg/day. Single cell necrosis was characterised by death of individual hepatocytes throughout the liver, with limited inflammatory cell involvement. There was a dose-related reduction in the level of glycogen vacuolation in animals given 250 or 500 mg/kg/day. Glycogen vacuolation was characterised by generally perinuclear, clear, variably sized, indistinctly defined, vacuoles. Finally, increased mitosis was present in animals from all groups given beta-damascone. The greatest severity was present in animals given 250 mg/kg/day, and the lowest incidence was present in animals given 500 mg/kg/day. Increased mitosis was characterised by an increase, above the normal low background incidence, of mitotic figures within the liver parenchyma. Collectively, these findings indicate that the test animals were systemically exposed to beta-damascone.

The negative control had a 0.11 % average rate of micronucleated polychromatic erythrocytes (MNPCE) and a ratio of 50.2 % polychromatic erythrocytes (PCE); 125 mg/kg beta-damascone treatment group had a MNPCE rate of 0.09 % and PCE ratio of 49.17 %; 250 mg/kg treatment group had 0.09 % MNPCE rate and 52.30 % PCE ratio; 500 mg/kg treatment group showed 0.06 % MNPCEs and 37.63 % PCE ratio. The positive control group resulted in 1.54 % MNPCEs and a 43.17 % PCE ratio (Beevers, 2013c). The group mean frequencies observed were similar to concurrent vehicle controls for all dose groups and also were within the historical control values (mean: 0.12 %). There was a reduction in PCE ratio at the highest dose level indicating bone marrow toxicity, which demonstrates target organ exposure. These results showed that there was no statistically significant increase in micronucleus induced with beta-damascone under these test conditions when compared to negative control group. In addition, there were no statistically significant differences among

erythrocyte parameters examined in this study. It was concluded that beta-damascone did not induce micronucleated erythrocytes in rat bone-marrow cells following administration by oral gavage.

The Comet assay in the liver tissue did not reveal cytotoxicity, necrosis or apoptosis in the hepatocytes as assessed by cloud and halo analysis. Hepatocytes of rats dosed with beta-damascone were evaluated for % tail intensities and tail moments ( $\pm$  standard error of the mean, SEM); 125 mg/kg beta-damascone group had  $2.45 \pm 0.13$  % tail intensity and  $0.27 \pm 0.02$  % tail moment; 250 mg/kg group had  $2.99 \pm 0.31$  % tail intensity and  $0.33 \pm 0.03$  tail moment; 500 mg/kg group had  $2.93 \pm 0.24$  % tail intensity and  $0.31 \pm 0.03$  tail moment that were similar to concurrent vehicle controls (tail intensity of  $2.67 \pm 0.26$  % and  $0.29 \pm 0.03$  tail moment) and fell within the testing laboratories historic control range for vehicle controls (0.3 - 8.15 % tail intensity and 0.04 - 0.81 tail moment). The Comet arm of this study confirms that beta-damascone did not induce DNA damage in the liver under the conditions of this study (Beevers, 2013c).

In a satellite study the slides from the duodenum tissue samples collected in the above study (Beevers, 2013c) were analysed for Comet tailing effects (Beevers, 2013b). Duodenum cells of rats dosed with beta-damascone were evaluated for % tail intensities and tail moments ( $\pm$  standard error of the mean, SEM); 125 mg/kg beta-damascone group had  $2.01 \pm 0.43$  % tail intensity and  $0.32 \pm 0.03$  % tail moment; 250 mg/kg group had  $1.47 \pm 0.15$  % tail intensity and  $0.16 \pm 0.02$  tail moment; 500 mg/kg group had  $2.03 \pm 0.19$  % tail intensity and  $0.19 \pm 0.02$  tail moment that were similar to concurrent vehicle controls (tail intensity of  $2.24 \pm 0.43$  % and  $0.23 \pm 0.04$  % tail moment) and fell within the testing laboratories historic control range for vehicle controls (0.3 - 8.15 % tail intensity and 0.04 - 0.81 tail moment). The duodenum Comet arm of this study confirms that beta-damascone did not induce DNA damage in the duodenum under the conditions of this study. The vehicle control data were within historical control ranges (95 % reference range: 0.77 to 8.32 % for tail intensity and 0.08 to 1.15 for tail moment) and the positive control induced a clear increase in DNA damage. The study was therefore confirmed as valid. There was no evidence of duodenum toxicity as would be suggested by increases in clouds or halo cells.

The % tail intensity and tail moment at all dose levels were very similar to the concurrent vehicle control, thus confirming there is no test article-related DNA damage. The additional tissue sample analysis for comet tailing showed a negative result for this study (Beevers, 2013b).

The results from the combined *in vivo* micronucleus induction study and Comet assay show that orally administered beta-damascone did not induce micronucleated erythrocytes in rat bone-marrow cells nor genotoxic events in liver and duodenum of rats.

## CONCLUSION

FGE.213 concerned 26 substances, corresponding to subgroup 2.7 of FGE.19 (see Table 1). Twenty-three of the substances are  $\alpha,\beta$ -unsaturated alicyclic ketones [FL-no: 07.008, 07.010, 07.014, 07.041, 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 07.083, 07.089, 07.108, 07.109, 07.117, 07.118, 07.119, 07.120, 07.127, 07.136, 07.168, 07.200 and 07.224] and three are precursors for such ketones [FL-no: 02.106, 09.305 and 09.525].

For 11 substances [FL-no: 07.047, 07.056, 07.057, 07.075, 07.076, 07.080, 7.117, 07.118, 07.119, 07.120 and 07.168] the Panel have ruled out concerns regarding genotoxicity in FGE.213

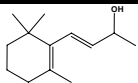
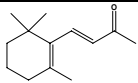
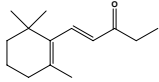

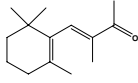

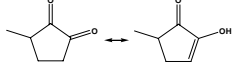
In the present opinion FGE.213Rev1, new data have been evaluated for the representative of the remaining substances. More specifically, data for beta-ionone [FL-no: 07.008], beta-damascone [FL-no: 07.083], maltol [FL-no: 07.014], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109]. All these studies are fully compliant with current guidelines, and stand in contrast to earlier studies previously evaluated in FGE.213.

The combined evidence from *in vitro* and *in vivo* genotoxicity data for the selected representative substances beta-ionone [FL-no: 07.008], beta-damascone [FL-no: 07.083], nootkatone [FL-no: 07.089] and 2,6,6-trimethylcyclohex-2-en-1,4-dione [FL-no: 07.109] does not indicate a genotoxic potential. Therefore, these substances and the nine substances that they represent, being [FL-no: 02.106, 07.010, 07.041, 07.108, 07.127, 07.136, 07.200, 07.224 and 09.305] could be evaluated through the procedure.

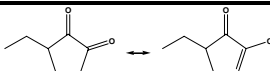
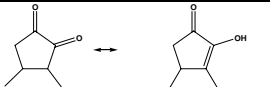
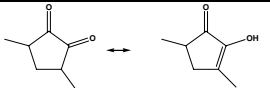
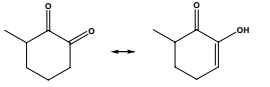
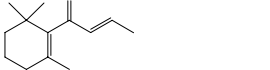
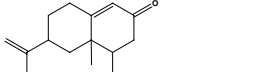
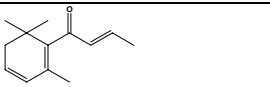
For maltol [FL-no: 07.014] and maltyl isobutyrate [FL-no: 09.525], the Panel concluded that the concern for genotoxicity could not be ruled out.

**SUMMARY OF SPECIFICATION FOR SUBSTANCES IN FGE.213REV1 (JECFA, 1998; JECFA, 2000; JECFA, 2005A; JECFA, 2005B; JECFA, 2009B)**

**Table 2:** Specification Summary of the Substances in the FGE. 213Rev1

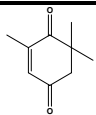
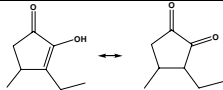
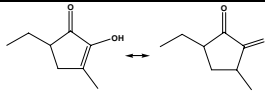
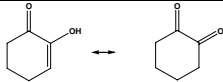
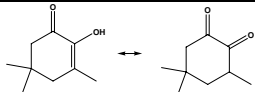
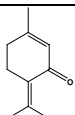
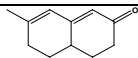
FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility <sup>(a)</sup> Solubility in ethanol <sup>(b)</sup>	Boiling point, °C <sup>(c)</sup> Melting point, °C ID test Assay minimum	Refrac. Index <sup>(d)</sup> Spec.gravity <sup>(e)</sup>
02.106 392	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		3625  22029-76-1	Liquid C <sub>13</sub> H <sub>22</sub> O 194.32		107 (4 hPa)  IR 92 %	1.499 0.927-0.933
07.008 389	beta-Ionone		2595 142 14901-07-6	Liquid C <sub>13</sub> H <sub>20</sub> O 192.30	Insoluble 1 ml in 3 ml 70% alcohol	239  IR 95 %	1.517-1.522 0.940-0.947
07.010 399	Methyl-beta- ionone		2712 144 127-43-5	Liquid C <sub>14</sub> H <sub>22</sub> O 206.33		238-242  IR 88 %	1.503-1.508 0.930-0.935
07.014 1480	Maltol		2656 148 118-71-8	Solid C <sub>6</sub> H <sub>6</sub> O <sub>3</sub> 126.11	Very slightly soluble Soluble	159-162 NMR 98 %	n.a. n.a.
07.041	beta-Isomethylionone		4151 650 79-89-0	Solid C <sub>14</sub> H <sub>22</sub> O 206.32	Freely soluble	334 62  95 %	n.a. n.a.
07.047 1481	Ethyl maltol		3487 692 4940-11-8	Solid C <sub>7</sub> H <sub>8</sub> O <sub>3</sub> 140.14	Soluble Soluble	89-93 NMR 99 %	n.a. n.a.
07.056 418	3-Methylcyclopentan-1,2-dione		2700 758 80-71-7	Solid C <sub>6</sub> H <sub>8</sub> O <sub>2</sub> 112.13	1 g in 72 ml water 1 g in 5 ml 90% alcohol	104-108 IR 95 %	

**Table 2:** Specification Summary of the Substances in the FGE. 213Rev1

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility <sup>(a)</sup> Solubility in ethanol <sup>(b)</sup>	Boiling point, °C <sup>(c)</sup> Melting point, °C ID test Assay minimum	Refrac. Index <sup>(d)</sup> Spec.gravity <sup>(e)</sup>
07.057 419	3-Ethylcyclopentan-1,2-dione		3152 759 21835-01-8	Solid C <sub>7</sub> H <sub>10</sub> O <sub>2</sub> 126.16	Miscible	78-80 (5 hPa) 36-43 IR 90 %	1.47-1.48 (25°) 1.060-1.066
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		3268 2234 13494-06-9	Solid C <sub>7</sub> H <sub>10</sub> O <sub>2</sub> 126.16		66 (1 hPa) 68-72 IR 98 %	
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		3269 2235 13494-07-0	Solid C <sub>7</sub> H <sub>10</sub> O <sub>2</sub> 126.16	Insoluble	87-93 MS 98 %	
07.080 425	3-Methylcyclohexan-1,2-dione		3305 2311 3008-43-3	Solid C <sub>7</sub> H <sub>10</sub> O <sub>2</sub> 126.16	Insoluble	69-72 (1 hPa) 57-63 IR 98 %	
07.083 384	beta-Damascone		3243 2340 23726-92-3	Liquid C <sub>13</sub> H <sub>20</sub> O 192.30	1 ml in 10 ml 95%	67-70 IR 90 %	1.496-1.501 0.934-0.942 (20°)
07.089 1398	Nootkatone		3166 11164 4674-50-4	Liquid C <sub>15</sub> H <sub>22</sub> O 218.35	Slightly soluble Soluble	73-103 (1 hPa) NMR 93 %	1.510-1.523 1.003-1.032
07.108 387	beta-Damascenone		3420 11197 23696-85-7	Liquid C <sub>13</sub> H <sub>18</sub> O 190.28	1 ml in 10 ml 95% alcohol	60 IR 98 %	1.508-1.514 0.945-0.952 (20°)

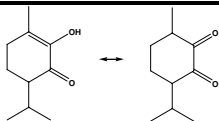
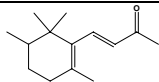
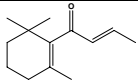
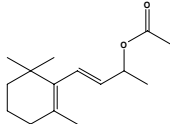
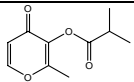
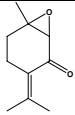


**Table 2:** Specification Summary of the Substances in the FGE. 213Rev1

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility <sup>(a)</sup> Solubility in ethanol <sup>(b)</sup>	Boiling point, °C <sup>(c)</sup> Melting point, °C ID test Assay minimum	Refrac. Index <sup>(d)</sup> Spec.gravity <sup>(e)</sup>
07.109 1857	2,6,6-Trimethylcyclohex-2-en-1,4-dione		3421 11200 1125-21-9	Solid C <sub>9</sub> H <sub>12</sub> O <sub>2</sub> 152.2	Slightly soluble Soluble	222 23-28 IR NMR 98 %	n.a. n.a.
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		3453 11077 42348-12-9	Liquid C <sub>8</sub> H <sub>12</sub> O <sub>2</sub> 140.18	Slightly insoluble Miscible	NMR 99 %	1.481-1.487 1.055-1.061
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		3454 11078 53263-58-4	Liquid C <sub>8</sub> H <sub>12</sub> O <sub>2</sub> 140.18	Slightly soluble Soluble	NMR 99 %	1.478-1.484 1.053-1.060
07.119 424	2-Hydroxycyclohex-2-en-1-one		3458 11046 10316-66-2	Solid C <sub>6</sub> H <sub>8</sub> O <sub>2</sub> 112.13	Soluble Soluble	53 (3 hPa) 35-38 IR 99.3 %	
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		3459 11198 4883-60-7	Solid C <sub>9</sub> H <sub>14</sub> O <sub>2</sub> 154.21	Slightly soluble Soluble	90-100 (20 hPa) 88 IR 99 %	
07.127 757	p-Mentha-1,4(8)-dien-3-one		3560 11189 491-09-8	Liquid C <sub>10</sub> H <sub>14</sub> O 150.22	Insoluble Miscible	233 MS 95 %	1.472-1.478 0.976-0.983
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnaphthalen-2(3H)-one		3715 34545-88-5	Solid C <sub>11</sub> H <sub>14</sub> O 162.23	Insoluble Soluble	n.a. 36-37 IR 99 %	n.a. n.a.



**Table 2:** Specification Summary of the Substances in the FGE. 213Rev1

FL-no JECFA-no	EU Register name	Structural formula	FEMA no CoE no CAS no	Phys.form Mol.formula Mol.weight	Solubility <sup>(a)</sup> Solubility in ethanol <sup>(b)</sup>	Boiling point, °C <sup>(c)</sup> Melting point, °C ID test Assay minimum	Refrac. Index <sup>(d)</sup> Spec.gravity <sup>(e)</sup>
07.168 2038	2-Hydroxypiperitone		4143 490-03-9	Solid C <sub>10</sub> H <sub>16</sub> O <sub>2</sub> 168.24	Slightly soluble Freely soluble	233 82 NMR MS 98 %	n.a. n.a.
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		79-70-9	Liquid C <sub>14</sub> H <sub>22</sub> O 206.33	Practically insoluble or insoluble Freely soluble	108 (2 hPa) MS 95 %	1.515-1.521 0.943-0.949
07.224	tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one		3243 2340 23726-91-2			90 %	
09.305 1409	beta-Ionyl acetate		3844 10702 22030-19-9	Liquid C <sub>15</sub> H <sub>24</sub> O <sub>2</sub> 236.35	Insoluble Soluble	120 (3 hPa) NMR 92 %	1.474-1.484 0.934-0.944
09.525 1482	Maltyl isobutyrate		3462 10739 65416-14-0	Liquid C <sub>10</sub> H <sub>12</sub> O <sub>4</sub> 196.20	Insoluble Soluble	100 (0.01 hPa) IR 96 %	1.493-1.501 1.140-1.153
16.044 1574	Piperitenone oxide		4199 10508 35178-55-3	Solid C <sub>10</sub> H <sub>14</sub> O <sub>2</sub> 166.22	Soluble Soluble	25 NMR MS 95 %	n.a. n.a.

(a): Solubility in water, if not otherwise stated.

(b): Solubility in 95 % ethanol, if not otherwise stated.

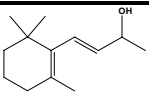
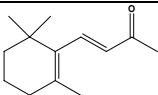
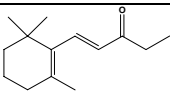
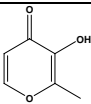
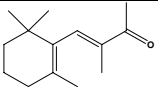
(c): At 1013.25 hPa, if not otherwise stated.

(d): At 20°C, if not otherwise stated.

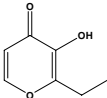
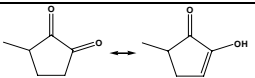
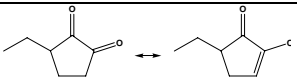
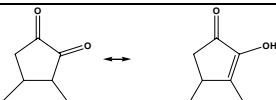
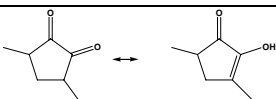
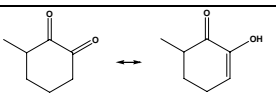
(e): At 25°C, if not otherwise stated.

# **SUMMARY OF SAFETY EVALUATION APPLYING THE PROCEDURE (JECFA, 1999; JECFA, 2001; JECFA, 2006A; JECFA, 2006B; JECFA, 2009A)**

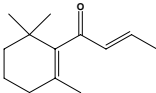
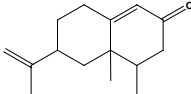
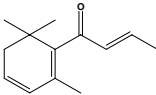
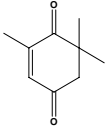
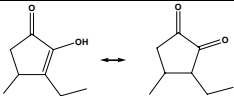
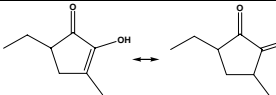
**Table 3:** Summary of Safety Evaluation Applying the Procedure

FL-no JECFA-no	EU Register name	Structural formula	MSDI <sup>(a)</sup> (µg/capita/ day)	Class <sup>(b)</sup> Evaluation procedure path <sup>(c)</sup>	Outcome on the named compound <sup>(d) or (e)</sup>	EFSA conclusion on the named compound (genotoxicity)
02.106 392	4-(2,2,6-Trimethyl-1-cyclohexenyl)but-3-en-2-ol		0.73 0.1	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.008 389	beta-Ionone		130 100	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.010 399	Methyl-beta- ionone		5.4 0.2	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.014 1480	Maltol		3060 2898	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could not be ruled out. Additional data requested.
07.041	beta-Isomethylionone		0.011	Not evaluated by the JECFA		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.

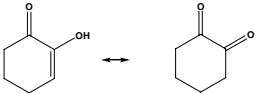
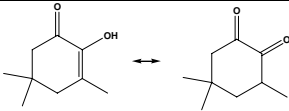
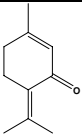
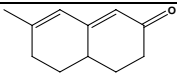
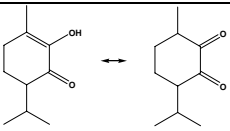
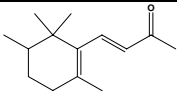
**Table 3:** Summary of Safety Evaluation Applying the Procedure

FL-no JECFA-no	EU Register name	Structural formula	MSDI <sup>(a)</sup> (µg/capita/ day)	Class <sup>(b)</sup> Evaluation procedure path (c)	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
07.047 1481	Ethyl maltol		1580 6692	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.83Rev1. No safety concern at the estimated level of intake based on the MSDI approach.
07.056 418	3-Methylcyclopentan-1,2-dione		570 710	Class II A3: Intake above threshold, A4: Not endogenous, A5: Adequate NOAEL exists	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.057 419	3-Ethylcyclopentan-1,2-dione		32 23	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		30 2	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		35 29	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.080 425	3-Methylcyclohexan-1,2-dione		1.3 8	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.

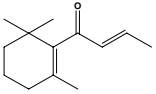
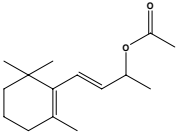
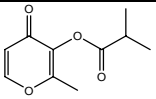
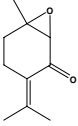
**Table 3:** Summary of Safety Evaluation Applying the Procedure

FL-no JECFA-no	EU Register name	Structural formula	MSDI <sup>(a)</sup> (µg/capita/ day)	Class <sup>(b)</sup> Evaluation procedure path (c)	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
07.083 384	beta-Damascone		37 10	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.089 1398	Nootkatone		130 20	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.87Rev2.
07.108 387	beta-Damascenone		73 5	Class I B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.109 1857	2,6,6-Trimethylcyclohex-2-en-1,4-dione		50	Class II No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.09Rev5.
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		ND 0.17	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		ND 0.38	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.

**Table 3:** Summary of Safety Evaluation Applying the Procedure

FL-no JECFA-no	EU Register name	Structural formula	MSDI <sup>(a)</sup> (µg/capita/ day)	Class <sup>(b)</sup> Evaluation procedure path (c)	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
07.119 424	2-Hydroxycyclohex-2-en-1-one		0.049 0.76	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		1.2 2	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Evaluated by JECFA before 2000. No EFSA consideration required.
07.127 757	p-Mentha-1,4(8)-dien-3-one		0.012 0.01	Class II B3: Intake below threshold, B4: Adequate NOAEL exists	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE57Rev1.
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnaphthalen-2(3H)-one		ND 0.04	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.87Rev2.
07.168 2038	2-Hydroxypiperitone		0.0012	Class III A3: Intake below threshold	(d)	Evaluated in FGE.213, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.11Rev2. No safety concern at the estimated level of intake based on the MSDI approach.
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		0.012	Class I No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.

**Table 3:** Summary of Safety Evaluation Applying the Procedure

FL-no JECFA-no	EU Register name	Structural formula	MSDI <sup>(a)</sup> (µg/capita/ day)	Class <sup>(b)</sup> Evaluation procedure path (c)	Outcome on the named compound (d) or (e)	EFSA conclusion on the named compound (genotoxicity)
07.224	tr-1-(2,6,6-Trimethyl-1-cyclohexen-1-yl)but-2-en-1-one		100	No evaluation		Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.12Rev5.
09.305 1409	beta-Ionyl acetate		ND 9	Class I A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could be ruled out. Can be evaluated using the Procedure in FGE.73Rev3. MSDI based on USA production figure.
09.525 1482	Maltyl isobutyrate		20 38	Class II A3: Intake below threshold	(d)	Evaluated in FGE.213Rev1, genotoxicity concern could not be ruled out. Additional data are requested.
16.044 1574	Piperitenone oxide		0.012 0.2	Class III A3: Intake below threshold	(d)	Evaluated in FGE.213, additional genotoxicity data required. The substance is not supported by the Industry any longer. No further evaluation.

(a): EU MSDI: Amount added to food as flavour in (kg / year) x 10E<sup>9</sup> / (0.1 x population in Europe (= 375 x 10E6) x 0.6 x 365) = µg/capita/day.

(b): Thresholds of concern: Class I = 1800 µg/person/day, Class II = 540 µg/person/day, Class III = 90 µg/person/day.

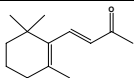
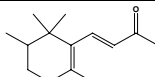
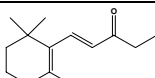
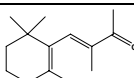
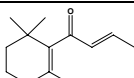
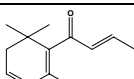
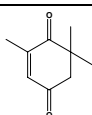
(c): Procedure path A substances can be predicted to be metabolised to innocuous products. Procedure path B substances cannot.

(d): No safety concern based on intake calculated by the MSDI approach of the named compound.

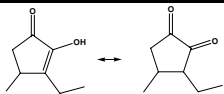
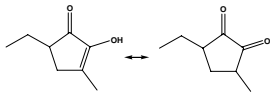
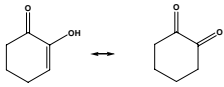
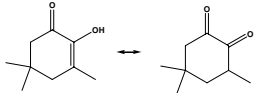


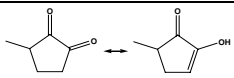
(e): Data must be available on the substance or closely related substances to perform a safety evaluation.

## QSAR PREDICTIONS ON MUTAGENICITY IN FIVE MODELS FOR 22 KETONES FROM SUBGROUP 2.7

**Table 4:** QSAR Predictions on Mutagenicity for 22 Alicyclic Ketones from Subgroup 2.7

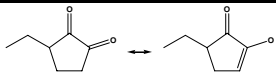
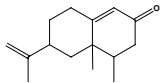
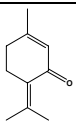
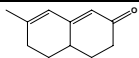
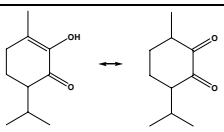
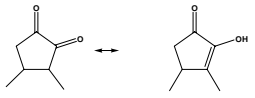
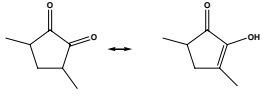
FL-no JECFA-no	EU Register name	Structural formula <sup>(a)</sup>	ISS Local Model Ames Test TA100 <sup>(b)</sup>	MultiCASE Ames test <sup>(c)</sup>	MultiCASE Mouse lymphoma test <sup>(d)</sup>	MultiCASE Chromosomal aberration test in CHO <sup>(e)</sup>	MultiCASE Chromosomal aberration test in CHL <sup>(f)</sup>
07.008 389	beta-Ionone		NEG	NEG	NEG	NEG	EQU
07.200	4-(2,5,6,6-Tetramethyl-1-cyclohexenyl)but-3-en-2-one		NEG	NEG	NEG	NEG	EQU
07.010 399	Methyl-beta- ionone		NEG	NEG	OD	OD	EQU
07.041	beta-Isomethylionone		NEG	EQU	NEG	NEG	NEG
07.083 384	beta-Damascone		OD	NEG	OD	OD	EQU
07.108 387	beta-Damascenone		OD	NEG	OD	OD	EQU
07.109	2,6,6-Trimethylcyclohex-2-en-1,4-dione		OD	NEG	OD	NEG	EQU

**Table 4:** QSAR Predictions on Mutagenicity for 22 Alicyclic Ketones from Subgroup 2.7

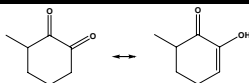
FL-no JECFA-no	EU Register name	Structural formula <sup>(a)</sup>	ISS Local Model Ames Test TA100 <sup>(b)</sup>	MultiCASE Ames test <sup>(c)</sup>	MultiCASE Mouse lymphoma test <sup>(d)</sup>	MultiCASE Chromosomal aberration test in CHO <sup>(e)</sup>	MultiCASE Chromosomal aberration test in CHL <sup>(f)</sup>
07.117 422	3-Ethyl-2-hydroxy-4-methylcyclopent-2-en-1-one		OD	NEG	NEG	OD	NEG
07.118 423	5-Ethyl-2-hydroxy-3-methylcyclopent-2-en-1-one		OD	NEG	NEG	NEG	NEG
07.119 424	2-Hydroxycyclohex-2-en-1-one		OD	OD	NEG	OD	NEG
07.120 426	2-Hydroxy-3,5,5-trimethylcyclohex-2-en-1-one		OD	NEG	NEG	OD	NEG
07.014 1480	Maltol		OD	OD	NEG	OD	POS
07.047 1481	Ethyl maltol		OD	OD	NEG	OD	POS
07.056 418	3-Methylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG



**Table 4:** QSAR Predictions on Mutagenicity for 22 Alicyclic Ketones from Subgroup 2.7

FL-no JECFA-no	EU Register name	Structural formula <sup>(a)</sup>	ISS Local Model Ames Test TA100 <sup>(b)</sup>	MultiCASE Ames test <sup>(c)</sup>	MultiCASE Mouse lymphoma test <sup>(d)</sup>	MultiCASE Chromosomal aberration test in CHO <sup>(e)</sup>	MultiCASE Chromosomal aberration test in CHL <sup>(f)</sup>
07.057 419	3-Ethylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.089 1398	Nootkatone		OD	NEG	NEG	NEG	POS
07.127 757	p-Mentha-1,4(8)-dien-3-one		OD	NEG	OD	NEG	NEG
07.136 1405	4,4a,5,6-Tetrahydro-7-methylnaphthalen-2(3H)-one		OD	NEG	NEG	NEG	OD
07.168 -	2-Hydroxypiperitone		OD	NEG	NEG	NEG	NEG
07.075 420	3,4-Dimethylcyclopentan-1,2-dione		OD	NEG	NEG	OD	NEG
07.076 421	3,5-Dimethylcyclopentan-1,2-dione		OD	NEG	NEG	NEG	NEG

**Table 4:** QSAR Predictions on Mutagenicity for 22 Alicyclic Ketones from Subgroup 2.7

FL-no JECFA-no	EU Register name	Structural formula <sup>(a)</sup>	ISS Local Model Ames Test TA100 <sup>(b)</sup>	MultiCASE Ames test <sup>(c)</sup>	MultiCASE Mouse lymphoma test <sup>(d)</sup>	MultiCASE Chromosomal aberration test in CHO <sup>(e)</sup>	MultiCASE Chromosomal aberration test in CHL <sup>(f)</sup>
07.080 425	3-Methylcyclohexan-1,2-dione		OD	NEG	NEG	OD	NEG

(a): Structure group 2.7:  $\alpha,\beta$ -unsaturated ketones.

(b): Local model on aldehydes and ketones, Ames TA100. (NEG: Negative; POS: Positive; OD\*: out of domain).

(c): MultiCase Ames test (OD\*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

(d): MultiCase Mouse Lymphoma test (OD\*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

(e): MultiCase Chromosomal aberration in CHO (OD\*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

(f): MultiCase Chromosomal aberration in CHL (OD\*: Out of domain; POS: Positive; NEG: Negative; EQU: Equivocal).

\* OD, out of applicability domain: not matching the range of conditions where a reliable prediction can be obtained in this model. These conditions may be physicochemical, structural, biological etc.

# GENOTOXICITY DATA (*IN VITRO*) CONSIDERED BY THE PANEL IN FGE.213

**Table 5:** Genotoxicity (*in vitro*)

Chemical Name [FL-no]	Test System	Test Object	Concentration	Reported Result	Reference	Comments <sup>(d)</sup>
beta-Ionone [07.008]	Gene mutation(preincubation)	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537	1-180 µg/plate	Negative <sup>(a)</sup>	(Mortelmans et al., 1986)	Valid.
	Gene mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537	3 mmol/plate	Negative <sup>(a)</sup>	(Florin et al., 1980)	Insufficient validity (spot test, not according to OECD guideline, methods and results insufficiently reported).
3-Methylcyclopentan-1,2-dione [07.056]	Reverse mutation	<i>S. typhimurium</i> TA1535	10 000 µg/plate	Negative <sup>(b)</sup>	(Heck et al., 1989)	Validity cannot be evaluated (result not reported in detail).
	Unscheduled DNA synthesis	Rat hepatocytes	500 µg/plate	Negative <sup>(b)</sup>	(Heck et al., 1989)	Validity cannot be evaluated (result not reported in detail).
Maltol [07.014]	Reverse Mutation	<i>S. typhimurium</i> TA100	4.44 µmol/plate (560 µg/plate)	Negative <sup>(c)</sup>	(Kim et al., 1987)	Insufficient validity (only one concentration was tested with only one bacterial strain without metabolic activation). The main purpose of the study was to investigate antimutagenic effects.
	Reverse Mutation	<i>S. typhimurium</i> TA98 and TA100	Up to 3 mg/plate (3000 µg/plate)	Positive <sup>(a)</sup>	(Bjeldanes and Chew, 1979)	Valid.
	Reverse Mutation	<i>S. typhimurium</i> TA92, TA98, TA100 and TA104	1.5 to 11 µmol/plate (189 to 1387 µg/plate)	Negative	(Gava et al., 1989)	Limited validity (data not reported in detail).
	Reverse Mutation	<i>S. typhimurium</i> TA1535, TA98, TA100 and TA1537	33 to 10 000 µg/plate	Positive <sup>(b)</sup>	(Mortelmans et al., 1986)	Valid.

	Reverse Mutation	<i>S. typhimurium</i> TA97 and TA102	0.1, 0.5, 1, 5, or 10 mg/plate (100, 500, 1000, 5000, or 10 000 µg/plate)	Weak Positive <sup>(a)</sup>	(Fujita et al., 1992)	Result is considered equivocal. Limited validity (the use of only two strains is not according to OECD guideline).
	DNA Damage (SOS Chromotest)	<i>Escherichia coli</i> PQ37	5 mM (631 µg/ml)	Negative	(Ohshima et al., 1989)	The test system used is considered inappropriate, due to insufficient validity.
	Sister Chromatid Exchange	Chinese hamster ovary cells	Up to 1.5 µmol/ml (12.6 to 189 µg/ml)	Positive <sup>(c)</sup>	(Gava et al., 1989)	Validity cannot be evaluated (insufficiently reported: number of cells analysed not reported. Statistical test used not reported). SCEs were reported as SCE per chromosome. Effect was less than twofold compared to control.
	Sister Chromatid Exchange	Human lymphocytes	Up to 1.0 mM (126.11 µg/ml)	Positive	(Jansson et al., 1986)	Validity cannot be evaluated. Relevance of test system for the evaluation of genotoxicity uncertain.
Ethyl maltol [07.047]	Reverse Mutation	<i>S. typhimurium</i> TA 1535, TA1537, TA1538, TA98 and TA100	5 concentrations up to cytotoxicity, or max. 3600 µg/plate	Negative <sup>(a)</sup>	(Wild et al., 1983)	Limited validity (result not reported in details, no TA102 or <i>E. Coli</i> ).
	Reverse Mutation	<i>S. typhimurium</i> TA98 and TA100	Up to 2 mg/plate (2000 µg/plate)	Positive <sup>(a)</sup>	(Bjeldanes and Chew, 1979)	Valid.

(a): With and without metabolic activation

(b): With metabolic activation

(c): Without metabolic activation

(d): Validity of genotoxicity studies:

Valid

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation)

Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system)

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

## GENOTOXICITY DATA (*IN VIVO*) CONSIDERED BY THE PANEL IN FGE.213

**Table 6:** Genotoxicity (*in vivo*)

Chemical Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments <sup>(a)</sup>
Maltol [07.014]	Micronucleus formation	ddY Mouse bone marrow cells	Intraperitoneal	125, 250, or 500 mg/kg	Positive	(Hayashi et al., 1988)	Valid. The induction of micronuclei was up to about 10-fold compared to control.
	Sex-linked Recessive Lethal Mutation	<i>Drosophila melanogaster</i>	Feeding	6000 ppm (6000 µg/ml)	Equivocal	(Zimmering et al., 1989)	Limited validity (only one exposure level tested). Test system considered of limited relevance.
	Sex-linked Recessive Lethal Mutation	<i>Drosophila melanogaster</i>	Feed	10 000 ppm (10 000 µg/ml)	Negative	(Mason et al., 1992)	Valid, however, test system considered of limited relevance.
	Sex-linked Recessive Lethal Mutation	<i>Drosophila melanogaster</i>	Injection	0.2 – 0.3 µl, 10 000 ppm (10 000 µg/ml)	Negative	(Mason et al., 1992)	Valid, however, test system considered of limited relevance.
Ethyl maltol [07.047]	Micronucleus formation	NMRI Mouse bone marrow cells	Intraperitoneal	420, 700, or 980 mg/kg	Negative	(Wild et al., 1983)	Limited validity (injected twice; only analysis at one time point; no PCE/NCE ratio reported).
	Micronucleus formation	NMRI Mouse bone marrow cells	Intraperitoneal	980 mg/kg	Negative	(Wild et al., 1983)	Limited validity (single injection, analysis at three time points, no PCE/NCE ratio reported).
	Sex-linked Recessive Lethal Mutation (Basc test)	<i>Drosophila melanogaster</i>	Feed	14, 25 or 50 mM	Negative	(Wild et al., 1983)	Limited validity (limited reporting, test system considered of limited relevance).

(a): Validity of genotoxicity studies:

Valid.

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).

Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

## CARCINOGENICITY STUDIES CONSIDERED BY THE PANEL IN FGE.213

**Table 7:** Carcinogenicity Studies

Chemical Name [FL-no]	Species; Sex No./Group	Route	Dose levels	Duration	Results	Reference	Comments <sup>(a)</sup>
Ethyl maltol [07.047]	Rats; Male, Female 25/sex/group	Diet	0, 50, 100 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences Females: No increase in tumour incidences	(Gralla et al., 1969)	Valid. The study was performed before the introduction of OECD guidelines but is however considered valid. The NOAEL was 200 mg/kg bw/day, the highest dose tested.
3-Ethylcyclopentan-1,2-dione [07.057]	Rats; Male, Female 50/sex/group	Diet	0, 30, 80 and 200 mg/kg bw/day	2 years	Males: No increase in tumour incidences Females: No increase in tumour incidences	(King et al., 1979a)	Valid. The study was performed before the introduction of OECD guidelines but is however considered valid. The NOAEL was 200 mg/kg bw/day, the highest dose tested.

(a): Validity of genotoxicity studies:

Valid.

Limited validity (e.g. if certain aspects are not in accordance with OECD guidelines or current standards and / or limited documentation).

Insufficient validity (e.g. if main aspects are not in accordance with any recognised guidelines (e.g. OECD) or current standards and/or inappropriate test system).

Validity cannot be evaluated (e.g. insufficient documentation, short abstract only, too little experimental details provided).

# GENOTOXICITY DATA (*IN VITRO*) CONSIDERED BY THE PANEL IN FGE.213REV1

**Table 8:** Summary of Additional *in vitro* Genotoxicity Data for FGE.213Rev1

Chemical Name [FL-no:]	Test System <i>in vitro</i>	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
beta-Ionone [07.008]	Reverse Mutation	<i>S. typhimurium</i> TA98, TA100, TA102, TA1535 and TA1537	0.32-5000 µg/plate [1,2]	Negative	Ballantyne, 2011	Evidence of toxicity was observed in all strains at 1000 µg/plate and above in the absence and in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		<i>S. typhimurium</i> TA98, TA100, TA102, TA1535 and TA1537	10.24-1000 µg/plate [2,4] or [3,5]	Negative		Evidence of toxicity was observed in all strains at 1000 µg/plate in the absence and presence of S9-mix, and in most cases these toxic effects also extended down to concentrations of 160 or 400 µg/plate. Study design complied with current recommendations.
	Micronucleus Assay	Human peripheral blood lymphocytes	30-60 µg/ml [4,6] 80-120 µg/ml [5,6] 5-17.5 µg/ml [4,7]	Negative	Stone, 2011a	The top concentrations induced 50-60 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.
Maltol [07.014]	Reverse Mutation	<i>S. typhimurium</i> TA98, TA100 and TA102, TA1535 and TA1537	0.32-5000 µg/plate [1,2]	Negative	Ballantyne, 2012	Evidence of toxicity was observed in TA102 at 1000 and 5000 µg/plate in the absence of S9-mix and at 200 µg/plate and above in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		<i>S. typhimurium</i> TA98, TA100, TA1535 and TA1537	51.2-5000 µg/plate [2,4] or [3,5]	Negative		Toxicity was observed at 5000 µg/plate in strain TA100 only in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.

**Table 8:** Summary of Additional *in vitro* Genotoxicity Data for FGE.213Rev1

Chemical Name [FL-no:]	Test System <i>in vitro</i>	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
	Micronucleus Assay	<i>S. typhimurium</i> TA102	20.48-5000 µg/plate [2,4] or [3,5]	Negative	Whitwell, 2012	Evidence of toxicity was observed at 5000 µg/plate in the absence and presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
		Human peripheral blood lymphocytes	400-1262 µg/ml [4,6]	Equivocal		The top concentrations in the 3+21 hours treatments in the absence and presence of S9-mix induced, respectively, 24% and 19% of toxicity. The top concentration in the 24+0 hours treatment in the absence of S9-mix induced 57 % toxicity. There was evidence of micronuclei induction when tested for 3+21 hours in the presence of S9-mix, while in absence of S9-mix the data were considered equivocal. However, no induction of micronuclei was observed in the continuous exposure test. Study design complies with OECD Guideline 487.
			400-1262 µg/ml [5,6]	Positive		
			125-300 µg/ml [4,7]	Negative		
beta-Damascone [07.083]	Reverse Mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537 and TA102	0.32-5000 µg/plate [1,2]	Negative	Bowen, 2011b	Toxicity was observed at 1000 and/or 5000 µg/plate across all strains in the absence and presence of S-9; no clear evidence of toxicity in TA100 in the presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, either in the presence or absence of S9-mix.
		<i>S. typhimurium</i> , TA1535, TA1537 and TA102	78.13-2500 µg/plate [2,4] or [3,5]	Negative		Evidence of toxicity was observed at the highest three or four concentrations across all strains in the absence and presence of S9-mix. No statistically significant increase in revertant numbers was seen at any concentration, either in the presence or absence of S9-mix
		<i>S. typhimurium</i> TA98, TA100	156.3-5000 µg/plate [2,4] or [3,5]	Negative		
		<i>S. typhimurium</i> TA98	19.3-1250 µg/plate [3,5]	Negative		Evidence of toxicity was observed at the highest four concentrations in strain TA98 in the presence of S9-mix. No statistically significant increase in revertant numbers was seen at any



**Table 8:** Summary of Additional *in vitro* Genotoxicity Data for FGE.213Rev1

Chemical Name [FL-no:]	Test System <i>in vitro</i>	Test Object	Concentrations of Substance and Test Conditions	Result	Reference	Comments
						concentration, in the presence of S9-mix.
	Micronucleus Assay	Human peripheral blood lymphocytes	8-22 µg/ml [6,4] 12-18 µg/ml [6,5] 6-9 µg/ml [7,4]	Equivocal [6,4] Positive [6,5] Equivocal [7,4]	Stone, 2012	Positive result was obtained in the 3+21 hour treatment in the presence of S9-mix. Study design complies with OECD Guideline 487.
Nootkatone [07.089]	Reverse Mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537 and TA102	0.5-50 µg/plate [2,4] 1.5-150 µg/plate [2,5]  0.5-50 µg/plate [2,4] 0.5-150 µg/plate [3,5]	Negative	Marzin, 1998	Evidence of toxicity was observed at 50 µg/plate in all strains in the absence of S9-mix and at 150 µg/plate in all strains in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus Assay	Human peripheral blood lymphocytes	50-80 µg/ml [4,6] 160-185 µg/ml [5,6] 10-24 µg/ml [4,7]	Negative	Stone, 2011b	The top concentrations in all parts of the study induced > 50 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.
2,6,6-Trimethylcyclohex-2-en-1,4-dione [07.109]	Reverse Mutation	<i>S. typhimurium</i> TA98, TA100, TA1535, TA1537 and TA102	0.32-5000 µg/plate [1,2]   156.3-5000 µg/plate [2,4] or [3,5]	Negative   Negative	Bowen, 2011a	Evidence of toxicity was observed at 1000 and/or 5000 µg/plate in strains TA102 and TA1535 in the presence of S9-mix. Study design complied with current recommendations. Acceptable top concentration was achieved.  Evidence of toxicity was observed in TA102 at 2500 and 5000 µg/plate. Study design complied with current recommendations. Acceptable top concentration was achieved.
	Micronucleus Assay	Human peripheral blood lymphocytes	500-1522 µg/ml [4,6] 1000-1522 µg/ml [5,6] 300-550 µg/ml [4,7]	Negative	Lloyd, 2011	The top concentrations in the 3+21 hours in the absence and presence of S9-mix were 10 mM. The top concentration in the 24+0 hours in the absence of S9-mix induced 57 % toxicity. The MNBN cell frequencies in all treated cultures fell within the normal range. Study design complies with OECD Guideline 487.

[1] With and without S-9 metabolic activation; [2] Plate incorporation method; [3] Pre-incubation method; [4] Without S-9 metabolic activation; [5] With S-9 metabolic activation; [6] 3-hour incubation with 21-hour recovery period; [7] 24-hour incubation with no recovery period

## GENOTOXICITY DATA (*IN VIVO*) CONSIDERED BY THE PANEL IN FGE.213REV1

**Table 9:** Summary of Additional *in vivo* Genotoxicity Data Submitted for FGE.213Rev1

Name [FL-no]	Test System	Test Object	Route	Dose	Result	Reference	Comments
Maltol [07.014]	Micronucleus assay	Han Wistar Rat; M	Gavage	70, 350, 700 mg/kg bw/day [1]	Inconclusive	Beevers, 2013a	The average MNPCE appearance frequency and ratio of PCE at all dose levels fell within concurrent and historical control ranges. However, evidence of animal exposure was inconclusive. The study was performed in compliance with OECD Guideline 474.
	Comet assay	Han Wistar Rat; M	Gavage		Negative		Mean % tail intensity and mean tail moment were within historical control range at all test doses. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature.
beta-Damascone [07.083]	Micronucleus assay	Han Wistar Rat; M	Gavage	125, 250 and 500 mg/kg bw/day [1]	Negative	Beevers, 2013b,c	The average MNPCE appearance frequency and ratio of PCE at all dose levels fell within concurrent and historical control ranges. The study was performed in compliance with OECD Guideline 474.
	Comet assay	Han Wistar Rat; M	Gavage		Negative		Mean % tail intensity and mean tail moment were within historical control range at all test doses. The study was performed in compliance with recommendations of the Comet and IWGT workshop, Japanese Center for the Validation of Alternative Methods (JaCVAM) and current literature.

[1] Administered via gavage in 3 doses at times 0, 24 and 45 hours with sacrifice and harvest at 48 hours

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## ABBREVIATIONS

BW	Body Weight
CAS	Chemical Abstract Service
CEF	Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CHO	Chinese Hamster Ovary (cells)
CHL	Chinese Hamster Lung (cells)
CoE	Council of Europe
EC	European Commission
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization
FGE	Flavouring Group Evaluation
FISH	Fluorescence In Situ Hybridisation
FLAVIS (FL)	Flavour Information System (database)
GLP	Good Laboratory Practice
ID	Identity
IOFI	International Organization of the Flavor Industry
i.p.	intraperitoneal
IR	Infrared spectroscopy
JECFA	The Joint FAO/WHO Expert Committee on Food Additives
MNBN	MicroNucleated BiNucleate cells
MNPCE	Micronucleated Polychromatic Erythrocytes
MS	Mass Spectrometry
MSDI	Maximised Survey-derived Daily Intake
MTD	Maximum Tolerated Dose
NCE	NormoChromatic Erythrocytes
NMR	Nuclear Magnetic Resonance
No	Number
NOEL	No Observed Effect Level
NAOEL	No Observed Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
PCE	PolyChromatic Erythrocytes
(Q)SAR	(Quantitative) Structure Activity Relationship
RI	Replication Index
SCF	Scientific Committee on Food
WHO	World Health Organisation